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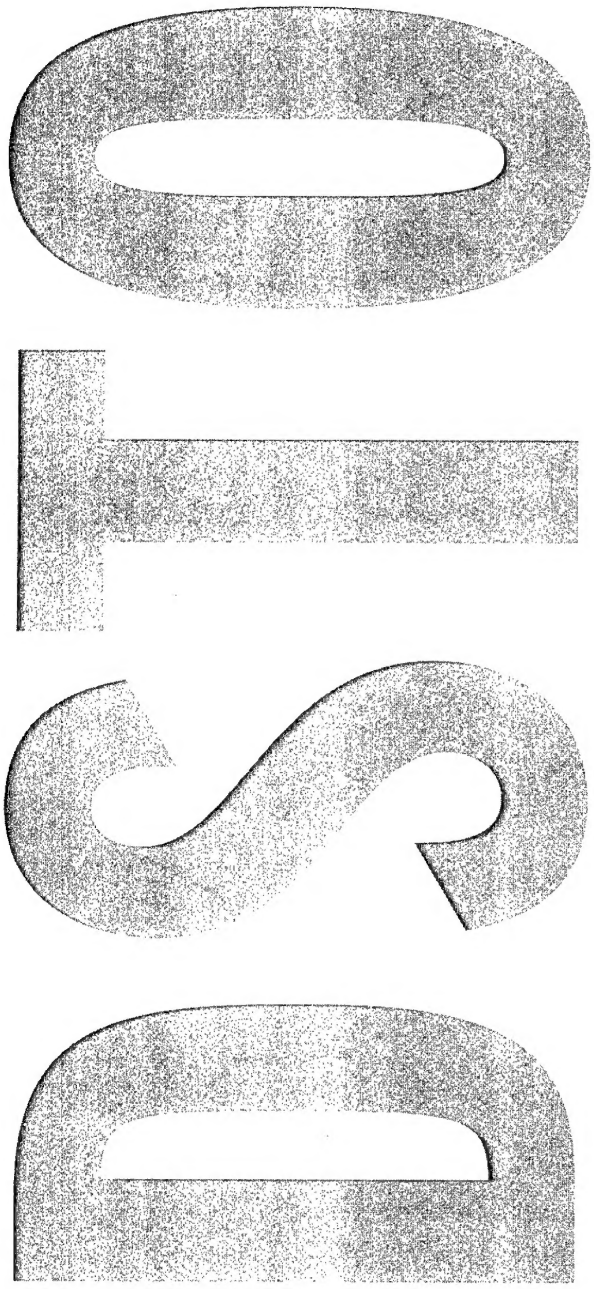

Adhesion and invasion of human lung epithelial cells by *Burkholderia pseudomallei*

Susan Shahin and David Proll

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ABSTRACT

Melioidosis is a potentially lethal infection that is endemic in Northern Australia and Southeast Asia. The causative bacterium, *Burkholderia pseudomallei,* is capable of adhering to and invading a number of mammalian cells. Lung epithelial cells are particularly susceptible following exposure by inhalation. In addition, since adhesion and subsequent invasion have been implicated as essential steps in the pathogenesis of invasive bacteria, inhibiting this mechanism may provide protection from disease. In this report we describe the development of an assay to investigate the adhesion by *B. pseudomallei* and subsequent invasion of human small airway epithelial cells *in vitro.* This assay will be used to assess the ability of specific molecules to inhibit the adhesion/invasion mechanism, thereby providing effective therapeutic measures against the infection.

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Executive Summary

Burkholderia pseudomallei causes the disease, melioidosis, which is endemic in Northern Australia, Southeast Asia and other potential areas of deployment for the ADF. The pathogenesis of the disease is variable with many organs being affected. The pulmonary form of the disease leading to lung abscess is fatal. Following a deliberate release of infectious agent in a bio-warfare scenario, the inhalational route is most likely the first point of entry. In the pathogenesis of invasive bacteria, an essential step is adhesion to host cells. *B. pseudomallei* is capable of adhering to and invading a number of mammalian cells. The mechanism involved in this interaction is poorly understood.

In this report we describe the development of an assay to investigate the adhesion and subsequent invasion of human small airway epithelial cells by *B. pseudomallei in vitro*. We have demonstrated the adhesion quantitatively by counting the number of adhering *B. pseudomallei* cells and qualitatively with both light and electron microscopy. The formation of adherent micro-colonies by *B. pseudomallei* was not apparent when the same assay was performed with a known non-adherent strain of *E. coli*. Invasion of the small airway epithelial cells by *B. pseudomallei* was also quantitated. This was achieved by treating the cells with an antibiotic to kill the adhering bacteria. In addition adhesion and invasion appear to be temperature dependent, which may be important in the regulation of the expression of virulence genes as the bacteria adapts to the environment of the human host.

As part of DSTO's ongoing research program, this assay will be used to assess the efficacy of antibodies raised against putative adhesion molecules, in inhibiting this adhesion and invasion. This will provide additional information for the development of effective vaccines and treatments, to protect ADF personnel from *B. pseudomallei* induced disease.

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Susan Shahin, PhD, joined the CBRN Defence Centre in 1998. Prior to DSTO, Susan worked at the University of Melbourne investigating the regulatory mechanisms of protein folding in yeast. Her work in DSTO includes the damaging effects of Sulphur mustard on mammalian cell DNA and development of rapid PCR techniques for the detection of biological warfare agents. Recently, her research has centred on the development of DNA vaccines against melioidosis.

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David Proll graduated from Monash University in 1993 with a B.Sc(Hons) and went on to complete his PhD in the department of Microbiology. His PhD studies focused on the replication of positive strand RNA viruses. After graduating from university he worked at the Eijkman Institute of Molecular Biology in Jakarta, Indonesia. Here, he investigated the application and development of DNA based vaccines against the parasite that causes Malaria. Upon returning to Australia in 2000, he was recruited by DSTO to initiate a research program investigating DNA vaccines for defence applications.

CONTENTS

1. Introduction

Burkholderia pseudomallei, the etiological agent of melioidosis, is endemic in subtropical areas including parts of Northern Australia and causes a severe invasive infection of humans and animals. Bacteria are capable of residing in cells for years after exposure before disease symptoms are manifested. This latency is particularly evident when a patient is immuno-compromised either through illness or advanced age. Relapse, even in patients treated with antibiotics, is common. Latency and reoccurrence are believed to result from the ability of *B. pseudomallei* to invade nonphagocytic host cells and to survive and replicate within phagocytes where antibiotics are less effective (Jones *et al,* 1996; Harley *et al,* 1998a; Harley *et al,* 1998b; Woods *et al,* 1999). The mechanism by which *B. pseudomallei* adheres to and enters epithelial cells is poorly understood.

Adhesion and subsequent invasion of mammalian cells have been implicated as essential steps in the pathogenesis of many intracellular facultative bacteria (Tomich *et al,* 2002; Cowan *et al,* 2000; Haager *et al,* 2003). The mechanisms involved in cell invasion include phagocytosis, bacteria-mediated endocytosis and mediation by specific membrane proteins (eg: invasin, extracellular adherence protein (EAP), flagellin and adhesin) (Chen *et al,* 2001; Tomich *et al,* 2002; Haggar *et al,* 2003; Inglis *et al,* 2003; Levy *et al,* 2003; Chau *et al,* 2003).

We are interested in the interaction of *B. pseudomallei* cells with human airway epithelial cells for a number of reasons. Following a deliberate release of infectious agent, infection by inhalation is of concern since the subsequent development of lung abscess is potentially fatal (Mary *et al,* 1995). We have targeted this interaction as a potential therapeutic measure by developing DNA vaccines against protein molecules that may by involved in the adhesion and invasion mechanism.

In this report we describe the development of an assay to investigate the adhesion and subsequent invasion of *B. pseudomallei* to human small airway epithelial cells *in vitro.* This assay may then be used to measure the efficacy of antibodies raised against putative adhesion and invasion molecules, in inhibiting this invasion. This will provide additional information for the development of effective vaccines and/or therapeutics to protect ADF personnel from *B. pseudomallei* infections.

2. Materials And Methods

2.1 Bacteria strains and growth conditions

The *B. pseudomallei* 08 strain used in this study is a clinical isolate from a severe case of melioidosis and was kindly provided by Professor Ifor Beacham (School of Health Science, Griffith University, Queensland). The *E. coli* DH5α strain was used for control purposes. Bacteria were grown on Luria-Bertani (LB) agar (Sigma) at 37^{0}C for 24 to 48 hours. Liquid cultures of LB broth inoculated with a single colony of *B. pseudomallei* were grown for 48 hours at either 37^{0}C or 30^{0}C with vigorous shaking. Antibiotics were added where appropriate. Bacterial cells were prepared for the adherence assay by washing once with phosphate buffered saline (PBS) and re-suspending the cell pellet in pre-warmed tissue culture medium (section 2.2).

The antibiotic resistance profile of the *B. pseudomallei* 08 strain was determined by plating a lawn of the bacteria onto LB agar in the presence of the following antibiotic disks: Choramphenicol (30 μg/ml), Kanamycin (50 μg/ml), Ciprofloxacin (2.5 μg/ml), Norfloxacin (10 μg/ml), Tetracycline (30 μg/ml), Gentamycin (120 μg/ml), Ampicillin (10 μg/ml) and Neomycin (30 μg/ml) (Oxoid, Australia). Plates were incubated for 48 hours at 30^{0}C or 37^{0}C. The diameter of the growth inhibition was measured in centimetres and termed the migratory inhibition constant (MIC).

2.2 Human small airway epithelial cells

Human small airway epithelial (SAE) cells were purchased from Clonetics (BioWhittaker, Inc. MD). Cells were grown at 37^{0}C with 5% CO_2 in basal medium (Clonetics) as recommended by the supplier. For each experiment a frozen aliquot of SAE cells was thawed, seeded in 75 cm^3 flasks and allowed to grow to 80-90 % confluency. Cells were dislodged with 0.025% trypsin/0.01% EDTA, counted using a heamocytometer and seeded into 24 well plates (5 x 10^5 cells/well) for adhesion and invasion assays and 6 well plates (1 x 10^6 cells/well) containing sterile glass coverslips for microscopy analysis. Cells were incubated overnight at 37^{0}C with 5% CO_2 before use.

2.3 Adhesion/Invasion assay

A flow diagram outlining the steps for both the adhesion and invasion assays is presented in Figure 1. Briefly, the medium from the SAE cells was removed and

SAE cells washed once with pre-warmed PBS. One ml of fresh medium containing either *B. pseudomallei* or *E. coli* DH5α cells, grown to stationary phase, at a multiplicity of infection (MOI) of approximately 10:1 (bacterial: epithelial cells) was added to the SAE cells and incubated for 2 hours at 37°C, 5% CO_2. The wells were washed 5 times with 1 ml of PBS prewarmed to 37°C to remove any unattached bacterial cells. To determine the total number of bacterial cells that had adhered and invaded, epithelial cells were dislodged and lysed with 0.1 % triton (v/v). Colony forming units (CFU) were determined by serial dilutions of the well contents in PBS and plated onto LB agar containing 10μg/ml ampicillin. The proportion of bacterial cells that had invaded was determined by adding fresh medium containing 150μg/ml tetracycline (Sigma) to kill any remaining extracellular bacteria. These cells were incubated for a further 2 hours at 37°C with 5% CO_2 in the presence of the antibiotic, washed, lysed and CFU determined by serial dilutions as described above. Serial dilutions of the original bacterial suspension were also made after completing the assay to determine the exact number of bacterial cells used for infection. The percentage of bacterial cells that adhered and invaded was calculated as the number of CFU/wells without tetracycline divided by the total number of cells added x 100. Likewise the percent of invading bacterial cells is expressed as a percentage of CFU of wells with tetracycline divided by the total x 100.

2.4 Microscopy

Human SAE cells grown in 6 well plates on glass coverslips to approximately 80 - 90 % confluency were washed once with PBS and 1 ml of basal medium containing either: stationary phase *B. pseudomallei* cells grown at 30°C or 37°C, or DH5α *E. coli* bacterial cells of the appropriate dilution were added. Bacterial cells were allowed to adhere for 2 hours at 37°C, 5% CO_2. Unattached bacterial cells were removed by washing 5 times with 1 ml of prewarmed PBS. The coverslips containing attached cells were air-dried, heat fixed and processed for light or electron microscopy.

Light microscopy: Cells were stained with either Gram stain or Giemsa stain (Sigma) following standard procedures (Murray *et al*, 1999). Some cells were also stained with safranin alone. Digital photographs were taken using a Canon PowerShot G5 mounted on a Ziess axiostar plus microscope.

Electron microscopy: The scanning electron microscopy was accomplished with the generous assistance of John Russell (Maritime Platforms Division, DSTO, Australia) and was conducted with a high resolution Field Emission Electron

Microscope (FESEM) using a LEO 1530 variable pressure (VP) with GEMINI column.

3. Results

3.1 Antibiotic resistance of *Burkholderia pseudomallei* 08 strain

To determine the antibiotic susceptibility profile of the *B. pseudomallei* 08 strain, a bacterial lawn was plated onto LB agar and incubated at 30°C or 37°C in the presence of the antibiotic disks listed in Table 1. The migratory inhibitory constant (MIC) is expressed in cm and is a measure of the diameter of the growth inhibition surrounding the antibiotic disk. A list of the antibiotics at the supplied concentrations and the measured MIC are summarized in Table 1. All of the listed antibiotics at the concentrations tested, with the exception of norfloxacin, inhibited the growth of *B. pseudomallei* 08 strain to varying degrees. However, since the disks contain varying antibiotic concentrations, a direct comparison of the degree of susceptibility of *B. pseudomallei* to the tested antibiotics cannot be made. The antibiotics chloramphenicol and tetracycline, both at the concentration of 30 μg/ml inhibited the growth to a greater degree. There was very little difference in the level of inhibition between the two incubation temperatures (Table 1) although the MIC for kanamycin was greater at 30°C than that exhibited at 37°C. Based on these results, tetracycline was selected to kill any exposed bacterial cells in our invasion assay. To ensure that all exposed bacteria are killed, a tetracycline concentration of 150 μg/ml was chosen which is 5 times that which was shown to inhibit growth to an MIC of 4 cm (Table 1).

3.2 Adhesion and invasion of human small airway epithelial cells by *Burkholderia pseudomallei*

The ability of *B. pseudomallei* to adhere to human SAE cells was investigated using the procedure summarised in Figure 1. *B. pseudomallei* cells were allowed to attach and/or invade human SAE cells for 2 hours, after which the unbound bacterial cells were removed by washing and the number of attached bacteria quantitated by calculating the number of CFU. Table 2 summarizes the results from a typical experiment and are expressed as the percentage of the total number of bacteria cells added. At an MOI of approximately 10, 13.55% of the *B. pseudomallei* cells grown at 30°C adhered to the human SAE cells. In contrast, when *B. pseudomallei* cells are grown at 37°C, the adhesion is reduced to 2.33%.

This value is similar to that observed for the non-adhering *E. coli* DH5α (3.88%) and most likely represents the level of non-specific binding.

The ability of *B. pseudomallei* to invade human SAE cells was also examined following tetracycline treatment to kill any residual bacterial cells that had not invaded (Figure 1). In a similar trend to the adhesive characteristics, 2.9% of *B. pseudomallei* cells incubated at 30ºC prior to the assay invaded the human SAE cells. This value correlates to approximately 30% of the adhering *B. pseudomallei* cells that proceeded to invade the SAE cells. This was at least 300 times greater than that exhibited by the non-invasive *E. coli* DH5α cells. In contrast, of those *B. pseudomallei* cells grown at 37ºC only 0.49% invaded the SAE cells. It should be noted that these values might be slightly higher due to the diffusive properties of tetracycline across the membrane of the epithelial cells. However, the effect is anticipated to be minimal due to the relatively short time of incubation in the presence of the antibiotic.

3.3 Microscopic analysis of *Burkholderia pseudomallei* adherence to human small airway epithelial cells.

To visualize the *B. pseudomallei* cells adhering to the human SAE cells, the adherence assay was performed with epithelial cells grown on microscope coverslips. Following the assay, cells were stained with safranin, Gram stain or Giemsa stain and examined by light microscopy. The representative images portrayed in Figure 2 demonstrate *B. pseudomallei* cells forming adherent microcolonies adjacent to the human SAE cells (Figure 2B and D) when stained with safranin. Images of human SAE cells alone (Figure 2A) processed under the same conditions are included for comparison and control purposes. To gain greater contrast between the SAE cells and the *B. pseudomallei* cells, preparations were stained with either Gram stain (Figure 2C and E) or Giemsa stain (Figure 2F). The cell bodies of the SAE cells retained more of the crystal violet dye of the Gram stain procedure than the *B. pseudomallei* cells (Figure 2C and E). In contrast, both the SAE cells and *B. pseudomallei* cells were stained to a similar degree with Giemsa stain (Figure 2F). Irrespective of staining procedure, few single bacteria were observed and the majority but not all of the bacteria clusters were adhering to epithelial cells (Figure 2C, E and F). However, many epithelial cells were also left free of bacteria (Figure 2C and F).

To assess whether growth temperature plays a role in the adherence of *B. pseudomallei* to the human SAE cells used in this study we compared the adhering properties of *B. pseudomallei* grown at both 30ºC and 37ºC. The non-adherent *E.coli* DH5α strain was also included as a control. Similar numbers of

the *B. pseudomallei* cells grown at 30°C or 37°C and *E. coli* DH5α cells were used in the adherence assays and slides stained with Giemsa (Figure 3). The nuclei of the human SAE cells were clearly distinguishable from the cytoplasm using this stain (Figure 3A). Neither the *B. pseudomallei* cells grown at 37°C (Figure 3C) nor the *E.coli* DH5α (Figure 3B) formed aggregating colonies compared to the *B. pseudomallei* cells grown at 30°C (Figure 3D). The arrows in panels B and C indicate single bacterial cells of *E.coli* DH5α and *B. pseudomallei* grown at 37°C sticking to the coverslip surface. Even though the Giemsa staining of the cytoplasm was faint we were unable to distinguish invading bacteria at the light microscopy level. To further demonstrate that the aggregating properties represent a significant step in the adhesion and not an aberrant function of the growth temperature, a smear of *B. pseudomallei* cells grown at the different temperatures was examined. There was no difference in the uniform appearance of the *B. pseudomallei* cells grown at 30°C compared to those grown at 37°C (results not shown).

The formation of adherent microcolonies by *B. pseudomallei* cells grown at 30°C when incubated with human SAE cells was also clearly demonstrated by scanning electron microscopy (Figures 4 and 5). When the cells were coated with gold and observed at low magnification (Figure 4), the perimeter of the human SAE cells was highlighted (Figure 4A). In a similar pattern to that observed by light microscopy, gold coating demonstrated the formation of adhering microcolonies of *B. pseudomallei* cells (Figure 4C, open arrow) both adjacent to epithelial cells (Figure 4C, closed arrow) and in isolated groups. Very few single adhering bacterial cells were observed. Using the secondary electron detector of the electron microscope and viewed at higher magnification (Figure 5), adherent *B. pseudomallei* cells were observed both in close proximity to the cell body (Figure 5A and C) and adjacent to the cell membrane (Figure 5B and D). The *B. pseudomallei* cells were coated more efficiently with gold under these conditions (Figure 5D). These micrographs may also suggest that the adherence is not random but involves some specific alignment with the epithelial cell extracellular matrix (Figure 5C).

4. Discussion

For many pathogens, adherence to the surface of host cells or the extracellular matrix is an important first step in the pathogenesis of infection. In the case of intracellular facultative bacteria such as *B. pseudomallei,* this bacterial colonisation is frequently followed by invasion. In this study we have developed an *in vitro* assay that demonstrates the adhesion and subsequent

invasion of human small airway epithelial cells by *B. pseudomallei*. The ability of *B. pseudomallei* to adhere to human epithelial cells has been documented elsewhere (Brown *et al*, 2002). However, these assays used A549 cells, which are a human alveolar epithelial carcinoma cell line. We have adapted this assay to demonstrate the adherence to a primary human small airway epithelial cell line by the *B. pseudomallei* 08 strain both quantitatively and qualitatively. The adherence of a stationary phase culture of *B. pseudomallei* to A549 cells reported by Brown *et al* (2002) was approximately 50% of the total number of bacterial cells added. This value is considerably higher than the 13% we have measured. A number of parameters could account for this difference. The variability between the results may be due to the different epithelial cell line that was used. In addition, subtle differences in the growth phase and ratios of the number of bacteria to SAE cells may influence the degree of adherence. Microscopically, the adhesion and micro-colony formation of *B. pseudomallei* in our system, correlated well with that presented by Brown *et al* (2002).

We were also interested in whether the *B. pseudomallei* 08 strain is capable of invading human small airway epithelial cells. Many researchers have used antibiotic protection assays to assess the invasion of mammalian cells. In these systems, bacterial cells outside the mammalian cells are exposed to and therefore susceptible to the antimicrobial action of the antibiotic. In contrast, the bacterial cells that have invaded mammalian cells are protected from the antibiotic and remain viable. Therefore, in order to perform the invasion assay it was necessary to determine the antibiotic susceptibility profile of the *B. pseudomallei* 08 strain. We discovered that the antibiotics kanamycin and gentamycin commonly used by other investigators to study invasion of *B. pseudomallei* (Stevens *et al*, 2003) and *B. cepacia* (Cieri *et al*, 2002) respectively, at the concentrations we tested were not as effective at inhibiting the growth of the *B. pseudomallei* 08 strain used in our study. The growth of the *B. pseudomallei* 08 strain was inhibited by the other aminoglycoside neomycin as reported in the literature (Murray *et al*, 1999) to a similar degree to both gentamycin and kanamycin. Using the antibiotic tetracycline, we have demonstrated that the *B. pseudomallei* 08 strain is capable of invading human small airway epithelial cells.

Brown *et al* (2002) also reported that the adherence of *B. pseudomallei* to the carcinoma A549 cell line was regulated by growth temperature. Consistent with their findings, the number of *B. pseudomallei* cells adhering to the small airway epithelial cells in our system decreased dramatically when the bacteria were cultured at the higher temperature of 37°C. In our assay, invasion was also significantly reduced when the bacteria are cultured at the higher temperature. However, Stevens *et al* (2003) demonstrated the invasion of *B. pseudomallei* 10276

strain, grown to stationary phase at 37°C, into HeLa cells. It must be noted that in this assay, the bacteria were centrifuged onto the HeLa cell monolayer and therefore the observed invasion may be an aberration induced through the centrifugation process. Alternatively, our results demonstrating that the temperature at which *B. pseudomallei* cells are grown affects both adhesion and invasion, may relate to the expression of specific protein receptor molecules at particular temperatures that are required for different host cell types.

Temperature dependent invasion has been demonstrated for a number of invasive bacteria. The temperature at which *Y. pestis* cultures are grown greatly enhances the bacteria's ability to invade HeLa cells (Cowan *et al*, 2000). This temperature dependence of *Y. pestis* invasion is due to the selective expression of phagocytic protective proteins. In *Y. enterocolitica* the expression of the *inv* gene coding for invasin is regulated by both growth temperature and growth phase (Badger *et al*, 2000). Similarly, growth to the stationary phase and therefore nutrient limitation correlates with the expression of virulence factors in the intracellular pathogen *L. pneumophilia* (Bachman and Swanson, 2001). Therefore, in the case of *B. pseudomallei* the lower temperature may reflect the optimal temperature required for the expression of appropriate genes necessary for adhesion and/or invasion and may mimic the temperature transition of the pathogen from the environment to the human host. Since *B. pseudomallei* is predominately a soil organism in endemic sub-tropical areas, one can postulate that at 30 degree temperatures, the pathogen is primed for the initial stages of infection.

Conversely, other invasive bacteria require different growth phase and temperature conditions for optimal *in vitro* invasion of host cells. For example, invasion of A549 epithelial cells by *B. cepacia spp.* (Cieri *et al*, 2002; Tomich et al, 2002), U937 derived macrophages (Martin & Mohr, 2000) and well differentiated human airway epithelial cells from lung transplant donors (Schwab *et al*, 2002) was demonstrated when the bacteria were cultured at 37°C. Similarly invasion of both fibroblasts and keratinocytes by *S. aureus* (Haager *et al*, 2003) was also apparent when bacteria are grown at 37°C prior to the assay. In these studies mid logarithmic growth cells were used and invasion efficiency was greatly enhanced when bacterial cells were centrifuged onto the mammalian cell monolayer.

There are many studies describing the involvement of numerous molecules in the adhesion /invasion mechanism. Attachment of *B. pseudomallei* to pharyngeal epithelial cells is mediated by the asialoganglioside GM1-GM2 receptor complex (Ahmed *et al*, 1999; Gori *et al*, 1999). Motility and functional flagella facilitate both adhesion and invasion (Brett *et al*, 1994; Brett and Woods 1996;

Tomich *et al*, 2002; Inglis *et al*, 2003; Chua *et al*, 2003). Stevens *et al* (2003) identified a *B. pseudomallei bopE* mutant, which displayed reduced invasion of HeLa cells. BopE is a putative type 111 secreted protein and the BopE facilitated invasion appeared to involve interference with the eukaryotic actin cytoskeleton. Jones *et al* (1997) described a transposon mutant of *B. pseudomallei* 1026B, which was deficient in invading A549 cells. Analysis of the nucleotide sequence revealed that the mutation disrupted the *irlR* gene encoding a putative two-component response regulator. Furthermore, Jones *et al* (1997) demonstrated that the mechanism utilised by bacteria to invade eukaryotic cells might not be necessary for the expression of virulence. In animal models of *B. pseudomallei* infection, there was no difference in the virulence of their invasion-deficient mutant compared to the parent strain. Other specific molecules such as the *Y. pestis* invasin (Cowan *et al*, 2000) and the *S. aureus* extracellular adherence protein (Hagger *et al*, 2003) play a significant role in both the adhesion and invasion processes of the respective bacterial species. Both the Hag protein (Holm *et al*, 2003) and the IgD binding protein (Forsgren *et al*, 2003) of *Moraxella catarrhalis* and the autotransporter Aae protein of *Actinobacillus actinomycetemcomitans* (Rose *et al*, 2003) display adhesion properties to human lung epithelial cells. These studies highlight the complexity of the mechanisms that invasive bacteria employ to adhere, invade and infect susceptible eukaryotic cells.

5. Concluding Remarks

Considering that *B. pseudomallei* is prevalent in areas of possible deployment, infections caused by the bacterium are a threat to ADF personnel particularly through inhalation. Currently there is no effective vaccine available for protection and existing therapy involves prolonged use of a combination of antibiotics. Therefore, an adhesion/invasion assay such as that described in this report may be used to assess and identify molecules that may be the targets of potential therapeutic measures.

Suitable candidates for DNA vaccines include protein molecules that may be involved in the mechanism of adhering and invading mammalian cells. Candidate vaccines against potential adhesins and other possible contributing proteins such as flagella are being developed. It is envisaged that the developed adhesion/invasion assay will be used to test the capability of antibodies, induced through immunisation with the engineered vaccines, in inhibiting the adherence of the *B. pseudomallei* cells.

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Appendix 1: Tables

Table 1: Antibiotic resistance and susceptibility profile of B. pseudomallei 08 strain.

Antibiotic	MIC# (cm)	
	37 °C	30 °C
Chloramphenicol (30 μg/ml)	3.3	2.3
Kanamycin (50 μg/ml)	1.7	2.2
Ciprofloxacin (2.5 μg/ml)	1.0	0.9
Norfloxacin (10 μg/ml)	0	0
Tetracycline (30 μg/ml)	4.0	4.0
Gentamycin (120 μg/ml)	1.5	1.2
Ampicillin (10 μg/ml)	0.8	0.8
Neomycin (30 μg/ml)	1.0	0.8

 # The migratory inhibitory constant was calculated by measuring the diameter of the growth inhibition surrounding the antibiotic disc in the bacterial lawn.

Table 2: Adhesion and invasion of B. pseudomallei and E.coli to cultured human SAE cells[a]

	Bps 08 (30°C)	Bps 08 (37°C)	DH5α
% adhesion	13.55 ± 1.06	2.33 ± 0.66	3.88 ± 0.96
% invasion	2.9 ± 0.61	0.49 ± 0.25	< 0.01

[a] The B. pseudomallei cells were incubated to stationary phase prior to the adhesion assay at the temperature indicated in brackets. The E.coli cells were only grown at 37°C. Adhesion is expressed as the proportion of the total of bacterial cells that remained attached following washing. The invasion is expressed as the proportion of total bacteria cells added that survived the tetracycline treatment. n=3.

Appendix 2: Figures

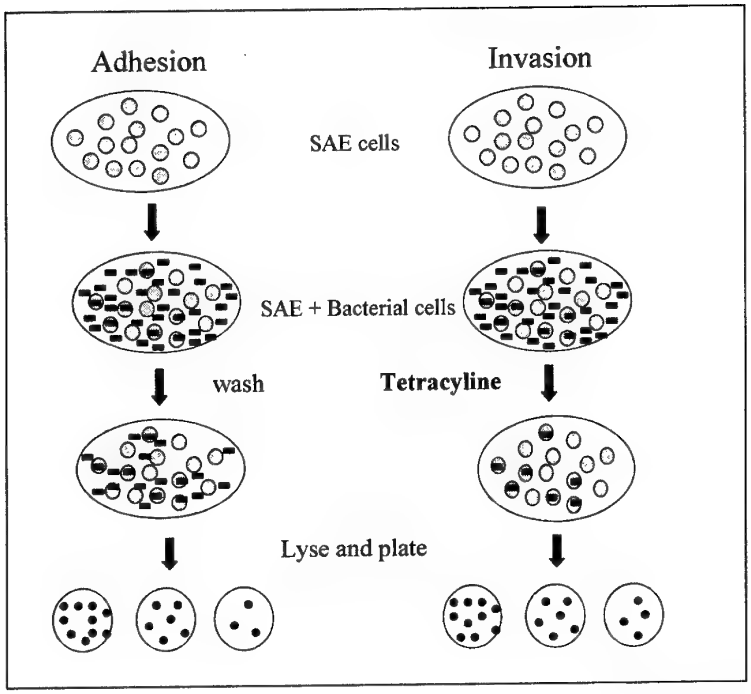

Figure 1: Schematic representation of the assay used to study the adhesion and invasion of human small airway epithelial cells by B. pseudomallei. *The number of bacterial cells that adhered and invaded is represented by those cells that survived washing. Invading bacteria protected by the epithelial cell membrane survived tetracycline treatment.*

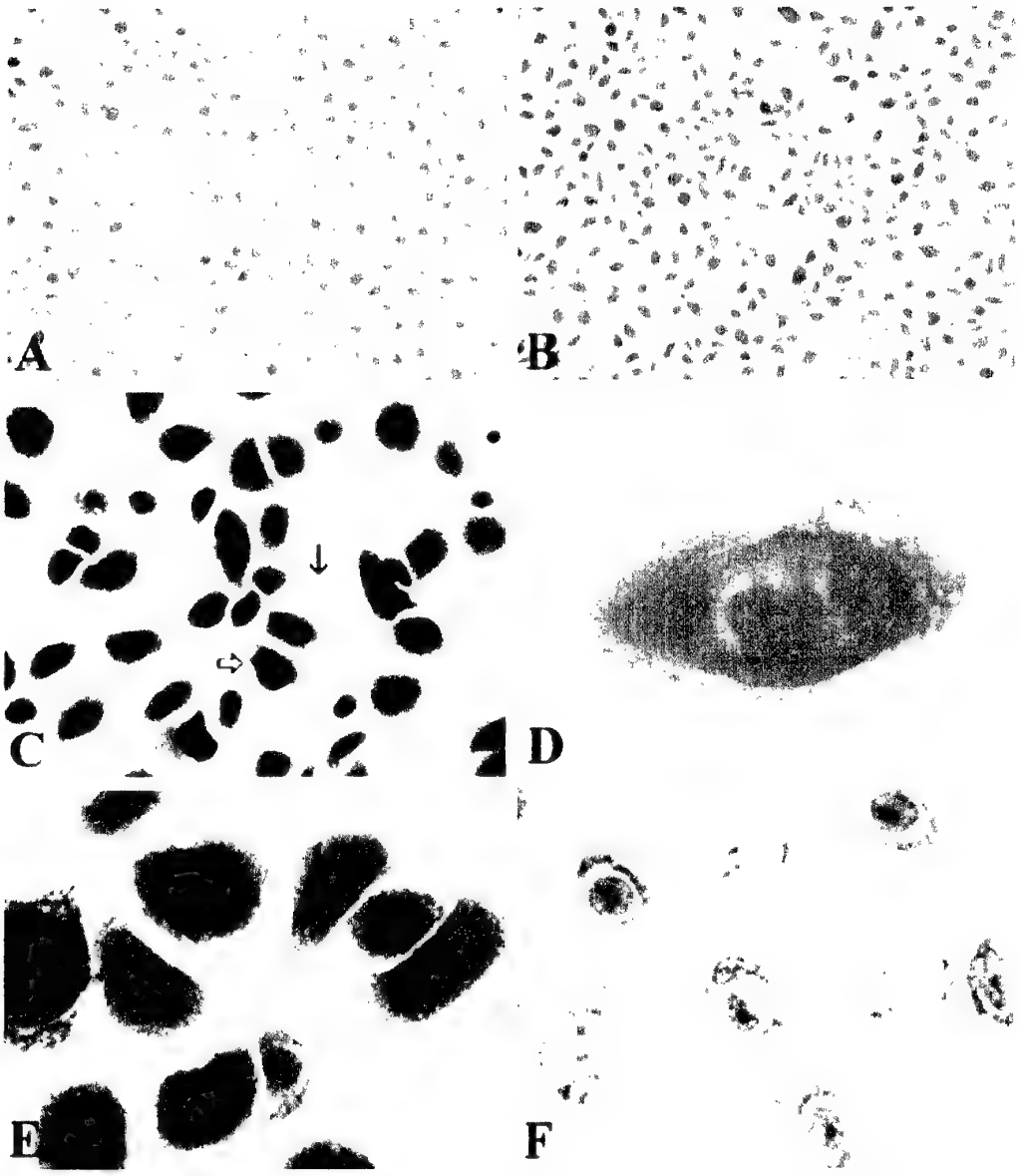

Figure 2: Light microscope images of B. pseudomallei adhering to human SAE cells. *The open and closed arrows indicate a SAE cell and adherent microcolonies of B. pseudomallei respectively. (A): human SAE cells. (B, C, D, E and F): adherence of B. pseudomallei cells grown at 30^{0}C to human SAE cells. Panels A, B and D stained with safranin and viewed with either a 10 X objective (A, B) or a 100 X objective (D). Panels C and E stained with Gram stain and observed with a 40 X objective (C) and a 100 X objective (E). Panel F stained with Giemsa and viewed with a 100 X objective.*

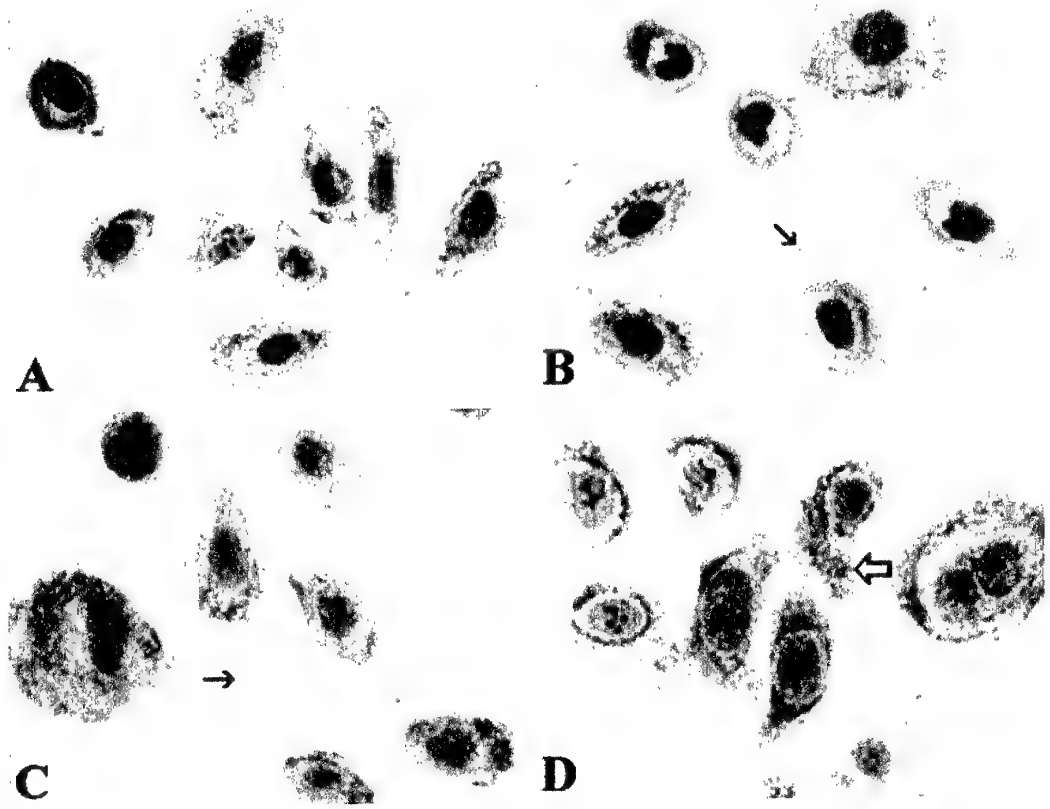

Figure 3: Light microscope images comparing the adherence of B. pseudomallei grown at either 30^{0}C or 37^{0}C to human SAE cells. *All preparations were stained with Giemsa and observed with a 100 X objective through 10 X eyepieces. (A): Human SAE cells. Adherence of E.coli DH5α (B), B. pseudomallei cultured at 30^{0}C (D) and 37^{0}C (C). The closed arrow in panels B and C indicate single E.coli and B. pseudomallei cells adhering to the coverslip surface. The open arrow in panel D indicates adherent microcolonies of B. pseudomallei cells in close proximity to the human SAE cells.*

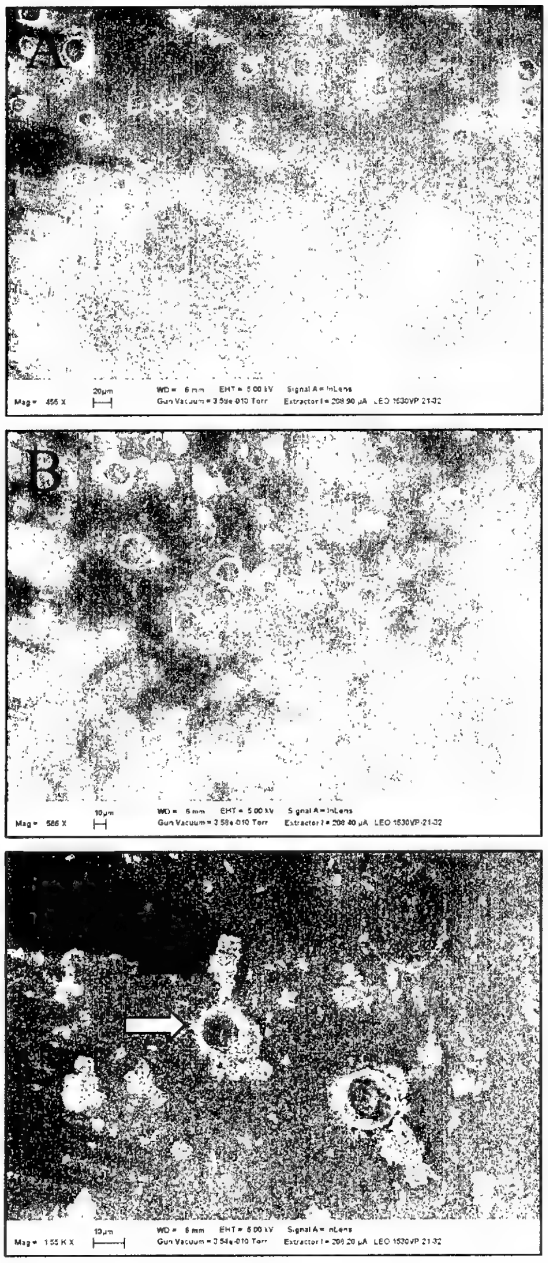

Figure 4: Adhesion of B. pseudomallei cells to human SAE cells observed by scanning electron microscopy at low magnification. *B. pseudomallei cells were incubated with SAE cells grown on coverslips. Preparations were coated with an evaporative layer of gold to give a contrast difference and micrographed using LEO's patented Inlens detector. The open and closed arrows indicate a SAE cell and adherent microcolonies of B. pseudomallei respectively (A): human SAE cells. (B, C): human SAE cells incubated with B. pseudomallei grown at 30^{0}C.*

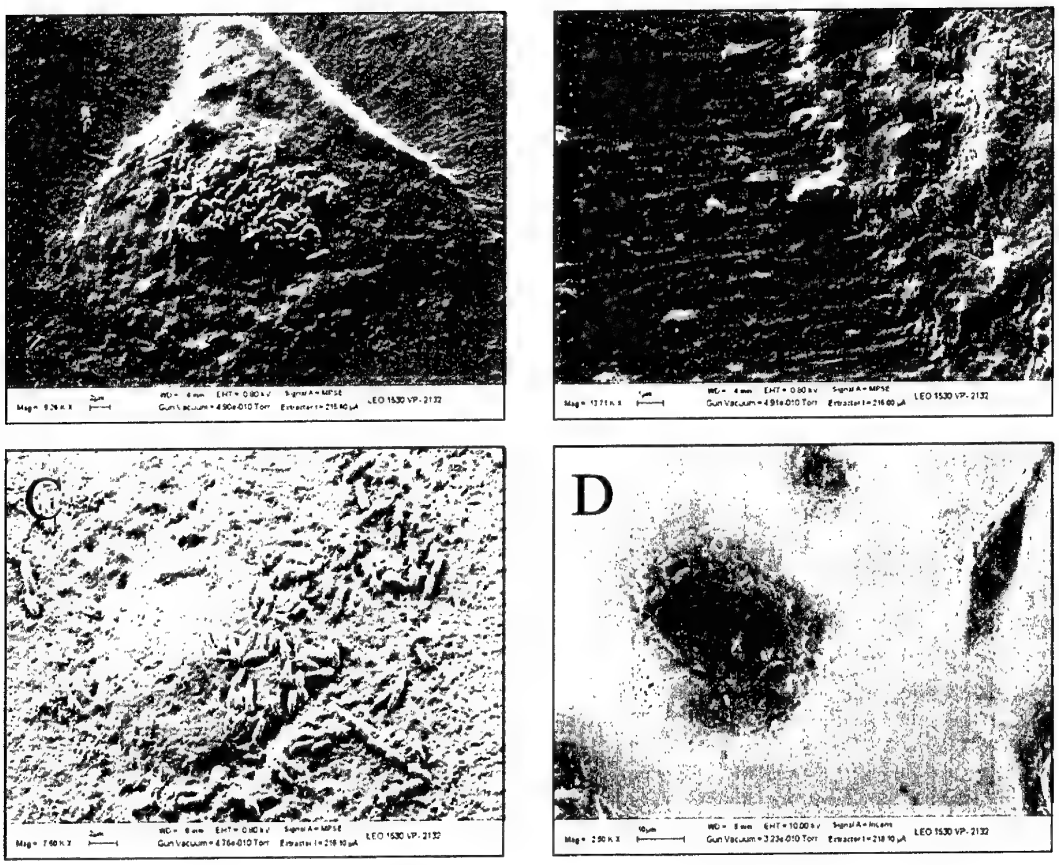

Figure 5: Adhesion of B. pseudomallei cells to human SAE cells observed by scanning electron microscopy at high magnification. *(A, B, C): slides without a metallic coating using LEO's multipurpose secondary electron detector (MPSE) at low kV (0.80 kV). (D): preparation coated with an evaporative layer of gold and micrographed using LEO's patented InLens.*

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Susan Shahin and David Proll

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19. ABSTRACT

Melioidosis is a potentially lethal infection that is endemic in Northern Australia and Southeast Asia. The causative bacterium, *Burkholderia pseudomallei*, is capable of adhering to and invading a number of mammalian cells. Lung epithelial cells are particularly susceptible following exposure by inhalation. In addition, since adhesion and subsequent invasion have been implicated as essential steps in the pathogenesis of invasive bacteria, inhibiting this mechanism may provide protection from disease. In this report we describe the development of an assay to investigate the adhesion by *B. pseudomallei* and subsequent invasion of human small airway epithelial cells *in vitro*. This assay will be used to assess the ability of specific molecules to inhibit the adhesion/invasion mechanism, thereby providing effective therapeutic measures against the infection.